

## SHORT COMMUNICATION

**Induction of activating transcription factor 3 by anoxia is independent of p53 and the hypoxic HIF signalling pathway**K Ameri<sup>1</sup>, EM Hammond<sup>1</sup>, C Culmsee<sup>2</sup>, M Raida<sup>3</sup>, DM Katschinski<sup>4</sup>, RH Wenger<sup>5</sup>, E Wagner<sup>2</sup>, RJ Davis<sup>6</sup>, T Hai<sup>7</sup>, N Denko<sup>1,9</sup> and AL Harris<sup>8,9</sup>

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**Solid tumors often have an inadequate blood supply, which results in large regions that are subjected to hypoxic or anoxic stress. Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that regulates much of the transcriptional response of cells to hypoxia. Activating transcription factor 3 (ATF3) is another transcription factor that responds to a variety of stresses and is often upregulated in cancer. We investigated the regulation of ATF3 by oxygen deprivation. ATF3 induction occurred most robustly under anoxia, is common, and it is not dependent on presence of HIF-1 or p53, but is sensitive to the inhibition of c-Jun NH<sub>2</sub>-terminal kinase activation and the antioxidant N-acetylcystein. ATF3 could also be induced by desferrioxamine but not by the mitochondrial poison cyanide or the nonspecific 2-oxoglutarate dioxygenase inhibitor dimethylxalylglycine. We also show that anoxic ATF3 mRNA is more stable than normoxic mRNA providing a mechanism for this induction. Thus, this study demonstrates that the regulation of ATF3 under anoxia is independent of 2-oxoglutarate dioxygenase, HIF-1 and p53, presumably involving multiple regulatory pathways. *Oncogene* (2007) 26, 284–289. doi:10.1038/sj.onc.1209781; published online 17 July 2006**

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Tumor hypoxia/anoxia is associated with a more aggressive clinical phenotype (Graeber *et al.*, 1996; Le *et al.*, 2004). It is thought that this phenotype is the

result of the hypoxic induction of a number of genes, the majority of which are regulated by hypoxia-inducible factor-1 (HIF-1) (Jiang *et al.*, 1996). In normoxia, 2-oxoglutarate-dependent dioxygenases (PHDs) hydroxylate HIF-1 $\alpha$ , which leads to interaction with the von Hippel–Lindau protein (pVHL), targeting HIF-1 $\alpha$  to proteasomal degradation. In hypoxia, hydroxylation does not occur, HIF-1 $\alpha$  is stabilized, it binds to HIF-1 $\beta$  and then transactivates target genes (Schofield and Ratcliffe, 2004).

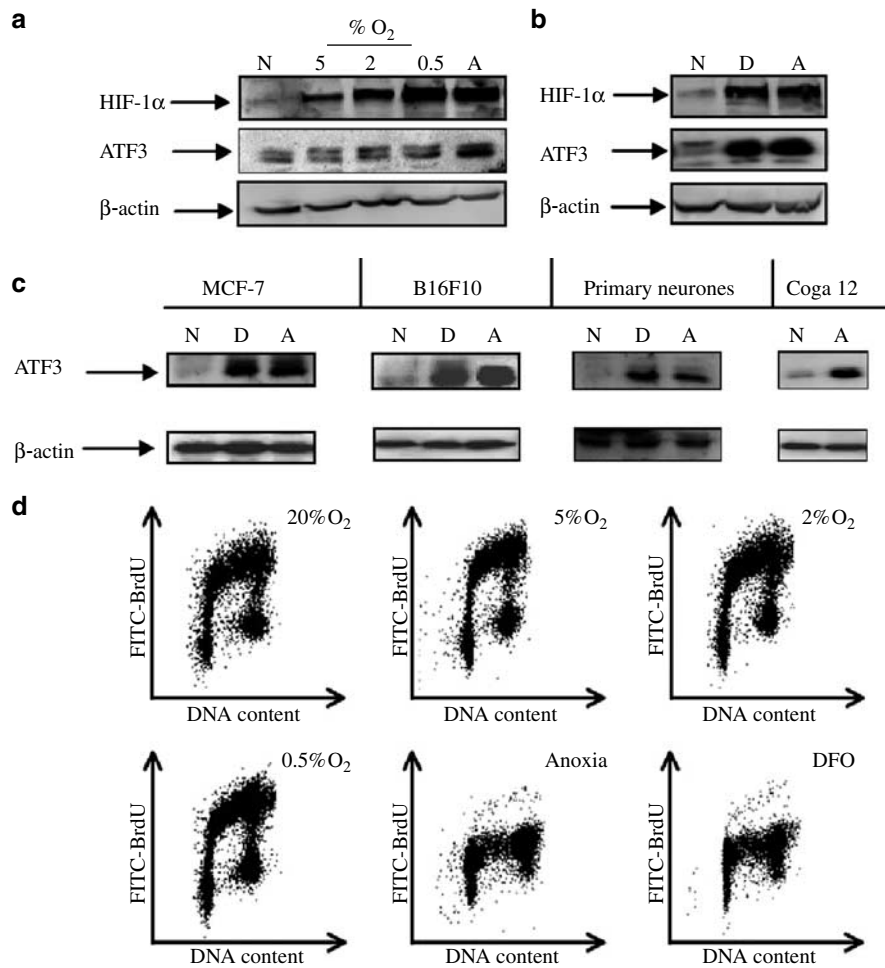
Anoxia has been shown to regulate gene expression in an HIF-1-independent manner (Ameri *et al.*, 2004; Blais *et al.*, 2004). To investigate the extent and mechanisms of HIF-independent gene regulation under low oxygen tensions, we considered activating transcription factor 3 (ATF3), a member of the ATF/cAMP-responsive element binding protein family of transcription factors, as a potential master regulator to alter the cellular transcriptional programs under hypoxia and anoxia for the following reasons. First, ATF3 is induced by a variety of stress signals (Hai *et al.*, 1999). Second, expression of ATF3 has been demonstrated to alter a variety of cellular processes relevant to cancer progression including cell cycle, cell death, angiogenesis and metastasis (Hai and Hartman, 2001; Okamoto *et al.*, 2006). Third, ATF3 expression can be regulated transcriptionally by a variety of signalling pathways or transcription factors, including nuclear factor kappa B (Hartman *et al.*, 2004), EGR-1 (Bottone *et al.*, 2005), by p53-dependent and -independent pathways (Amundson *et al.*, 1999; Fan *et al.*, 2002), and by the c-Jun NH<sub>2</sub>-terminal kinase (JNK) (Cai *et al.*, 2000), which is activated by MKK7 (Tournier *et al.*, 1997). In addition to promoter activation, ATF3 mRNA can also accumulate as a result of mRNA stabilization (Liang *et al.*, 1996). All of these pathways or regulators have been observed in the response of cells to hypoxia (Scott *et al.*, 1998; Alfranca *et al.*, 2002; Goldberg-Cohen *et al.*, 2002; Bottone *et al.*, 2005; Kaelin, 2005). Therefore, we investigated the regulation of ATF3 under conditions of oxygen deprivation.

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**Figure 1** Induction of ATF3 under hypoxia, anoxia and by DFO in various cell types. The human breast cancer cell line MCF-7 and murine B16-F10 melanoma cells were from American type culture collection (ATCC) and maintained as described before (Ameri *et al.*, 2004). Low passage Coga 12 colon cancer cells were previously isolated and prepared from human tumor samples as described (Vecsey-Semjen *et al.*, 2002). Embryonic murine cortical neurons (8–10 days cultures) were prepared and maintained as described, (Culmsee *et al.*, 2003). Cultures were incubated for 24 h in either DFO (200  $\mu$ M DFO) (Sigma, Taufkirchen, Germany) or in a humidified gas-regulated incubator-gloved box (InVivo2 400; Ruskin, Leeds, UK) at the indicated oxygen concentrations. For anoxic conditions, cells were placed in a humidified anaerobic work station with 5% H<sub>2</sub>, 5% CO<sub>2</sub> and 95% N<sub>2</sub> (Sheldon Co., Cornelius, OR, USA) and a palladium catalyst. Extracts were made directly in the chamber. Protein extracts were made with urea denaturing buffer added directly to the cells as described (Ameri *et al.*, 2004), they were quantitated and subjected to reducing sodium dodecyl sulphate–polyacrylamide (10–12%) gel electrophoresis and electroblotted to polyvinylidene difluoride. The rabbit polyclonal antibody to ATF3 (sc-188) was from Santa Cruz Biotechnology Inc. (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the mouse monoclonal antibody to HIF-1 $\alpha$  was from BD Transduction Laboratories (San Jose, CA, USA). (a) Immunoblot analysis shows that ATF3 protein is most robustly upregulated under anoxia compared to hypoxic levels of 5–0.5% O<sub>2</sub>, whereas HIF-1 $\alpha$  is stabilized at 5% O<sub>2</sub>. (b) Both DFO and anoxia upregulate ATF3 and HIF-1 $\alpha$  in MCF-7 cells. (c) Upregulation of ATF3 protein by anoxia and DFO is common in the cell lines tested. (d) Cells were incubated at the O<sub>2</sub> concentrations shown or in the presence of DFO for 16 h. One hour before harvesting, 10  $\mu$ M BrdU were added and BrdU incorporation and DNA content was examined as described (Hammond *et al.*, 2003). Experiments were performed at least three times. Immunoblot for  $\beta$ -actin served as a control (anti- $\beta$ -actin antibody was from Sigma, Taufkirchen, Germany). N = normoxia, A = anoxia, D = desferrioxamine (DFO).

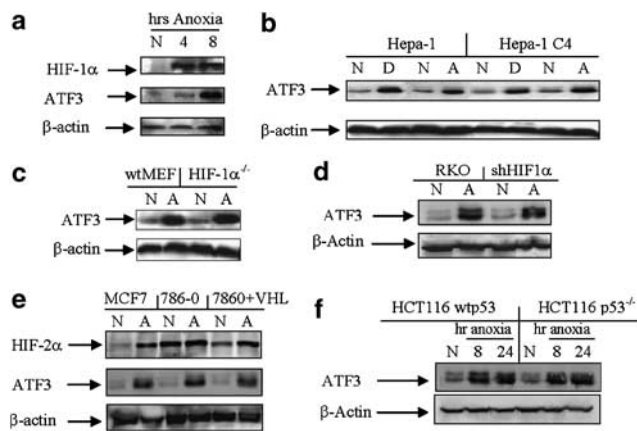
*Induction of ATF3 protein is a common cellular response to anoxia:* Figure 1a shows that ATF3 is robustly induced under anoxia (<0.1% O<sub>2</sub>) but not under hypoxic conditions (5–0.5% O<sub>2</sub>). These data suggest that HIF-1 $\alpha$  alone is not sufficient to induce ATF3 as HIF-1 $\alpha$  is stabilized in mild hypoxia. Figure 1b shows that the hypoxia-mimetic iron chelator desferrioxamine (DFO) can also induce ATF3 and HIF-1 $\alpha$  in MCF7 cells. Figure 1c demonstrates that ATF3 protein is upregulated under anoxia and by DFO in a wide

variety of different cells. One biologic difference between hypoxia and anoxia is that cells can continue to cycle in hypoxia whereas they arrest in anoxia (Papandreou *et al.*, 2005). Figure 1d shows that DFO can also cause an S-phase cell cycle arrest similar to anoxia. ATF3 can repress cyclin D1 gene expression and promote cell cycle arrest (Lu *et al.*, 2006).

*ATF3 induction does not require p53, VHL, HIF-2 $\alpha$  or HIF-1 $\alpha$ :* To determine the relative contribution of HIF-1 $\alpha$  to induction of ATF3 under anoxia, we first

compared a time course induction of ATF3 and HIF-1 $\alpha$  in anoxic MCF-7 cells. The time course study demonstrates that ATF3 protein is not induced coordinately with HIF-1 $\alpha$  (Figure 2a). Whereas HIF-1 $\alpha$  is induced within 4 h of anoxic incubation, ATF3 is induced after 8 h. This suggests that a greater level or duration of oxygen depletion is required to upregulate ATF3 protein as compared to HIF-1 $\alpha$  (Figure 2a). To investigate a direct role of HIF-1 $\alpha$  in regulation of ATF3 under anoxia, we examined ATF3 induction in three model systems deficient in HIF-1 $\alpha$ : (i) mouse embryonic fibroblasts (MEFs), (ii) RKO cells in which HIF-1 $\alpha$  was inhibited by shRNA and (iii) HIF-1 $\beta$  deficient Hepa-1 C4 cells. Figure 2b–d shows that ATF3 was induced in all three systems, demonstrating that HIF-1 $\alpha$  is not necessary for the induction of ATF3 under anoxia.

To further investigate the relationship between the HIF pathway and ATF3, we used 786-0 cells. The 786-0 cell line is deficient in VHL tumor suppressor and express HIF-2 $\alpha$  at a high constitutive level but do not express HIF-1 $\alpha$  at detectable levels. Stable VHL transfectants of 786-0 cells (786-0 + VHL) induce HIF-2 $\alpha$  in hypoxic/anoxic conditions (Maxwell *et al.*, 1999). Both 786-0 and 786-0 + VHL have low-level ATF3 in normoxia, but induced it in anoxia. Thus, a defect in



**Figure 2** Induction of ATF3 protein is not dependent on HIF-1 $\alpha$ , p53, a defect in pVHL or constitutive normoxic expression of HIF-2 $\alpha$ . (a) ATF3 protein is induced in MCF-7 cells at 8 h of anoxic incubation, whereas HIF-1 $\alpha$  is induced at 4 h. Hepa-1 and Hepa-1 C4 (HIF-1 $\beta$  deficient) cells and generation of HIF-1 $\alpha$ -deficient MEFs have been described before (Chilov *et al.*, 1999; Ryan *et al.*, 2000). DFO and/or anoxia induce ATF3 protein similarly in the genetically matched pairs of cells including (b) Hepa-1 cells and Hepa-1 C4, (c) wt and HIF-1 $\alpha$ -deficient MEFs (HIF-1 $\alpha$ <sup>-/-</sup>), (d) RKO (human colon carcinoma cells, American Type Culture Collection, ATCC) and HIF-1 $\alpha$  silenced RKO cells (described in Papandreou *et al.* (2006); designated as shHIF1 $\alpha$  in Figure 2d) and (e) 786-0 and 786-0 + VHL human renal cell carcinoma (Maxwell *et al.*, 1999). Owing to a VHL defect, HIF-2 $\alpha$  was induced constitutively in 786-0 cells under normoxia, whereas 786-0 + VHL cells and MCF7 cells did not express HIF-2 $\alpha$  in normoxia, but did induce HIF-2 $\alpha$  in anoxia. ATF3 was induced in all of the cells only under anoxia. (f) ATF3 was induced in both wtp53 and p53 null (p53<sup>-/-</sup>) HCT116 cells after 8 or 24 h of anoxia. Experiments were performed at least three times. N = normoxia, A = anoxia.

VHL and constitutive normoxic expression of HIF-2 $\alpha$  is also not sufficient to induce ATF3 protein (Figure 2e).

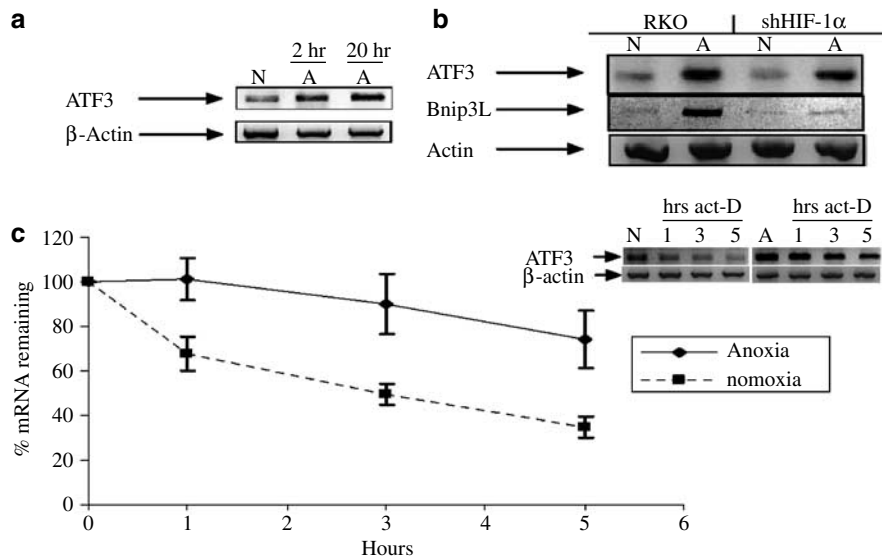
ATF3 has been considered as a p53 target based on CHIP-PET analysis (Wei *et al.*, 2006). We used the HCT116 paired p53 wild-type (wt) and null cell lines to investigate whether p53 contributes to ATF3 induction under anoxic conditions. Our results, Figure 2f, show that ATF3 was equally well induced under anoxia in both wt and p53 null HCT116 cells, indicating that ATF3 induction does not require p53. These results are consistent with other reports showing that p53 does not transactivate known targets in anoxia (Koumenis *et al.*, 2001; Hammond *et al.*, 2006).

*ATF3 mRNA is induced under anoxia independently of HIF-1 $\alpha$* : To determine whether ATF3 mRNA is induced under anoxia we performed a one-step reverse transcriptase–polymerase chain reaction (RT–PCR) using MCF-7 cells. Figure 3a shows that the steady-state level of ATF3 mRNA increases under anoxia after 2 h and persists at 20 h. The increase in ATF3 mRNA level (Figure 3a) precedes the increase in ATF3 protein level (Figure 2a), suggesting that increased ATF3 protein accumulation in anoxia could be due to a later post-transcriptional event. We do not rule out other non-HIF-1 pathways of increased protein translation that could also contribute to ATF3 accumulation under anoxia (Blais *et al.*, 2004). Figure 3b shows that the increase in ATF3 mRNA level under anoxia is independent of HIF-1 $\alpha$ .

*ATF3 mRNA is more stable under anoxia than under normoxia*: Induction of ATF3 mRNA could be through either increased transcription, decreased degradation or both. ATF3 mRNA has been shown to be stabilized by anisomycin and by amino-acid limitation (Liang *et al.*, 1996; Pan *et al.*, 2005). We investigated the stability of ATF3 mRNA under normoxia and anoxia by blocking new mRNA synthesis with actinomycin-D. As indicated in Figure 3c, ATF3 mRNA decays more slowly under anoxia than under normoxia. Addition of actinomycin-D to normoxic MCF-7 cells resulted in near 40% decline in ATF3 mRNA after 1 h, whereas anoxic ATF3 mRNA barely declined, suggesting a role for mRNA stability in response to anoxia. We also do not rule out an increased ATF3 promoter activity in induction of ATF3. Our preliminary results have shown that a 2-kb promoter region of ATF3 can activate reporter gene expression up to fourfold under anoxia (data not shown).

*The mitochondrial poison cyanide and the 2-oxoglutarate dioxygenase inhibitor dimethylxalylglycine do not induce ATF3*: We examined whether anoxia upregulated ATF3 due to blocked mitochondrial function. We used cyanide to mimic the anoxic block to mitochondrial electron transport. As demonstrated in Figure 4a, the anoxic induction of ATF3 or HIF-1 $\alpha$  protein in MCF7 cells or Coga cells could not be mimicked by using cyanide (CN), which suggests that ATF3 induction under anoxia was not dependent on arrest of the respiratory chain (Figure 4a).

In contrast, DFO could induce ATF3 (Figures 1b, c and 4a). This raises a paradox in which mild hypoxic



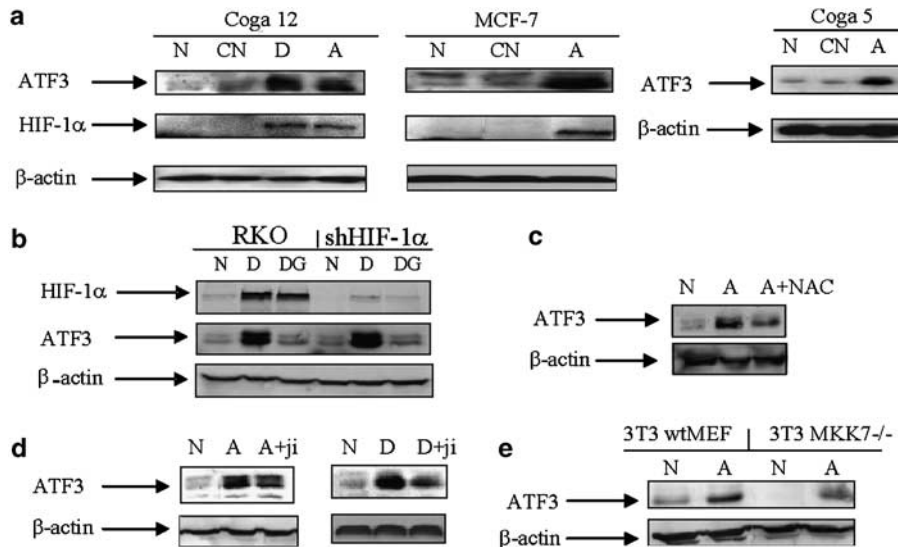
**Figure 3** ATF3 mRNA is upregulated under anoxia and is more stable than under normoxia. Total RNA was extracted from cells by using the Trizol (Invitrogen, Carlsbad, CA, USA) method according to the manufacturer's protocol. RT-PCR was carried out by using the superscript III one-step method (Invitrogen, Carlsbad, CA, USA). Primers were as follows: ATF3 forward primer CTGCAGAAAGAGTCGGAG, reverse primer TGAGCCCGGACAATACAC;  $\beta$ -actin forward primer TCACCGAGGCCCCCT GAACCCTA, reverse primer GGCAGTAATCTCCTTCTGCATCCT; Bnip3L forward ATGAATTCATGTCGTCGCCACC TAGTCG, reverse primer ATCTCGAGCTTCAGAAATTCTGCGGAGAAAATACCCCC. For message stability, cells were incubated in normoxia or anoxia for 20h and then 5  $\mu$ g/ml of actinomycin-D (Sigma-Aldrich, St Louis, MO, USA) was added directly to the cells and incubated for the indicated time points after which RNA was extracted. The quantity of mRNA was assessed by measuring the intensity of cDNA band on the gel with the Image Quant 5.2 program. (a) ATF3 mRNA was upregulated in MCF7 cells in anoxia. (b) ATF3 mRNA was upregulated under anoxia in both RKO and shHIF-1 $\alpha$  RKO cells after 20h whereas the HIF-1 $\alpha$  target gene Bnip3L was only upregulated in RKO cells. (c) As indicated, after addition of actinomycin-D, ATF3 mRNA under normoxia was less stable than ATF3 mRNA under anoxia. The graph illustrates the means  $\pm$  s.e. for three experiments. Results are normalized to the internal control  $\beta$ -actin. RT-PCR is a representative experiment. N = normoxia, A = anoxia, act.-D = actinomycin-D.

conditions are unable to induce ATF3, but the hypoxia-mimetic iron chelator DFO can. To determine whether the effects of DFO were through its ability to inhibit the PHDs, we tested another mechanistically distinct PHD inhibitor dioxygenase inhibitor dimethylxalylglycine (DMOG) for its ability to induce ATF3. RKO and HIF-1 $\alpha$ -silenced RKO cells were treated with DMOG and DFO. Figure 4b shows that whereas both DMOG (designated as DG, Figure 4b) and DFO can induce HIF-1 $\alpha$ , only DFO could induce ATF3. These results suggest that DFO's ability to induce ATF3 is not through inhibition of the PHDs, but through another mechanism. Our results are consistent with a recent report that ATF3 is induced by DFO but not by hypoxic conditions of 1% O<sub>2</sub> (Vengellur *et al.*, 2005). However, as this paper was completed, it was reported that ATF3 can be induced by DMOG (Elvidge *et al.*, 2006). This is not consistent with our findings, and considering DFO to be a hypoxia mimic via solely inhibition of PHDs can be misleading.

*Inhibiting JNK or adding of the free radical scavenger N-acetyl-l-cystein reduces the induction of ATF3 under anoxia:* The JNK pathway and free radicals have been previously suggested to contribute to gene regulation in hypoxia (Alfranca *et al.*, 2002; Kaelin, 2005). JNK and free radical signalling have also been suggested to be regulators of ATF3 expression (Cai *et al.*, 2000; Okamoto *et al.*, 2006). We therefore investigated the

effect of JNK inhibition and radical scavenging on induction of ATF3 protein under anoxia. Figure 4c shows that treating MCF7 cells with the antioxidant N-acetyl-l-cystein (NAC) reduced the induction of ATF3 under anoxia. Similarly, the JNK inhibitor SP600125 also reduced the levels of induction of ATF3 under anoxia and by DFO (Figure 4d). MKK7 can directly regulate JNK activity (Tournier *et al.*, 1997), and this signalling pathway has been previously suggested to be involved in regulating the expression of ATF3 (Cai *et al.*, 2000; Hartman *et al.*, 2004; Inoue *et al.*, 2004). We investigated the potential role of MKK7 in induction of ATF3 protein under anoxia. The results presented in Figure 4e demonstrate that 3T3 immortalized MKK7<sup>-/-</sup> MEFs had reduced levels of ATF3 expression under anoxia when compared to wtMEFs. We have also observed that primary MEFs lacking MKK7 completely fail to induce ATF3 under anoxia (data not shown, K Ameri unpublished observations). Thus, our data suggest that MKK7 and its downstream target JNK as well as free radicals are potentially involved in regulating ATF3 protein induction under anoxia.

The function of ATF3 in hypoxic/anoxic tumor sites remains unknown. ATF3 has been suggested to be critical in metastasis, and has also been reported to be proapoptotic as well as antiapoptotic (Ishiguro and Nagawa, 2000; Hai and Hartman, 2001; Francis *et al.*, 2004; Hartman *et al.*, 2004). As this paper was



**Figure 4** ATF3 is not induced by cyanide or DMOG, but induction under anoxia is reduced by addition of the free radical scavenger NAC and by JNK inhibition. **(a)** Potassium cyanide (5 mM, Sigma, Taufkirchen, Germany) and DFO (200  $\mu$ M) were added directly to the cells and incubated for 24 h in a 5% CO<sub>2</sub> incubator. Immunoblot analysis shows that low passage human Coga 12 and Coga 5 colon cancer cells and MCF-7 breast cancer cell line did not induce ATF3 (or HIF-1 $\alpha$ ) by cyanide but did by DFO. **(b)** RKO cells and shHIF-1 $\alpha$  RKO cells were incubated with 200  $\mu$ M DFO or 2 mM DMOG for 24 h. ATF3 protein was induced only by DFO and not by DMOG in both RKO and shHIF-1 $\alpha$ , whereas HIF-1 $\alpha$  was induced by both DFO and DMOG. **(c)** 10 mM NAC was added to the cells for 1 h in a 5% CO<sub>2</sub> incubator before treatment with anoxia for 24 h. Addition of NAC reduced the anoxic induction of ATF3. **(d)** For each separate experiment, a fresh batch of the JNK inhibitor SP600125 (Calbiochem Novabiochem, Bad Soden, Germany) was used. Fresh SP600125 was dissolved in 100% methanol and added to cells at a final concentration of 50  $\mu$ M for 1.5 h before treatment with DFO or anoxia for 24 h. As indicated, ATF3 induction by anoxia or DFO was reduced when cells were treated with the JNK inhibitor SP600125. **(e)** 3T3 immortalized MKK7<sup>-/-</sup> MEFs were described previously (Tournier *et al.*, 2001; Ventura *et al.*, 2004). Fibroblasts were seeded in 6 cm Petridishes, and incubated in a 5% CO<sub>2</sub> incubator at 37°C for 24 h until approximately 80% confluency. Cells were then subjected to anoxia for 20 h. As indicated, ATF3 protein was induced to a greater level in wtMEFs compared to MKK7<sup>-/-</sup> MEFs. Experiments were repeated three times. N=normoxia, A=Anoxia, D=desferrioxamine (DFO), DG=DMOG, CN=cyanide, Ji=JNK inhibitor, MEF=mouse embryonic fibroblast.

completed, it was reported that ATF3 stabilizes p53 upon genotoxic stress (Yan *et al.*, 2005), and also induces tubulogenic differentiation in endothelial cells and angiogenesis in diabetic angiopathy (Okamoto *et al.*, 2006). Thus, a potential role of ATF3 in modulating p53 and angiogenesis under anoxia should be investigated.

In summary, we demonstrate that the anoxic induction of ATF3 protein is independent of HIF-1 and p53,

but is reduced by MKK7 deficiency, JNK inhibition and the antioxidant NAC.

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